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Atypical protein kinase C activity is required for extracellular matrix degradation and invasion by Src-transformed cells

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Abstract

Atypical protein kinase C (aPKC) isoforms have been shown to mediate Src-dependent signaling in response to growth factor stimulation. To determine if aPKC activity contributes to the transformed phenotype of cells expressing oncogenic Src, we have examined the activity and function of aPKCs in 3T3 cells expressing viral Src (v-Src). aPKC activity and tyrosine phosphorylation were found to be elevated in some but not all clones of mouse fibroblasts expressing v-Src. aPKC activity was inhibited either by addition of a membrane-permeable pseudosubstrate, by expression of a dominant-negative aPKC, or by RNAi-mediated knockdown of specific aPKC isoforms. aPKC activity contributes to morphological transformation and stress fiber disruption, and is required for migration of Src-transformed cells and for their ability to polarize at the edge of a monolayer. The λ isoform of aPKC is specifically required for invasion through extracellular matrix in Boyden chamber assays and for degradation of the extracellular matrix in *in situ* zymography assays. Tyrosine phosphorylation of aPKC λ is required for its ability to promote cell invasion. The defect in invasion upon aPKC inhibition appears to result from a defect in the assembly and/or function of podosomes, invasive adhesions on the ventral surface of the cell that are sites of protease secretion. aPKC was also found to localize to podosomes of v-Src transformed cells, suggesting a direct role for aPKC in podosome assembly and/or function. We conclude that basal or elevated aPKC activity is required for the ability of Src-transformed cells to degrade and invade the extracellular matrix. Word count: 249.

Keywords

atypical protein kinase C; Src; transformation; migration; invasion; podosome

Introduction

The atypical subclass of the Protein Kinase C family of serine/threonine kinases (aPKCs) is comprised of two isoforms, the ζ isoform and a second isoform known as λ in the mouse and ι in humans; the two isoforms share over 70% amino acid identity (Newton, 1997). aPKCs are distinguished from other PKC family members in that they do not bind Ca^{2+} and are unresponsive to diacylglycerol (DAG) and phorbol esters (Newton, 1997). Like all PKC isoforms, aPKCs contain an auto-inhibitory pseudo-substrate motif at their amino-termini. Activation of aPKCs requires the phosphorylation of a Threonine residue (410 for the ζ isoform,

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403 for the λ isoform) within the activation loop (T-loop) by 3-phosphoinositide-dependent kinase 1 (PDK1) (Balendran et al., 2000; Le Good et al., 1998).

aPKCs have been implicated in the regulation of cellular transformation *in vitro* and in carcinogenesis *in vivo*. A constitutively active mutant of aPKC induces focus formation in 3T3 cells but does not promote their growth in soft agar (Le Good and Brindley, 2004). In 3T3 cells aPKC activity is also required for the ability of oncogenic Ras to produce foci and to induce morphological transformation and loss of actin stress fibers (Coghlan et al., 2000; Uberall et al., 1999). In addition, over-expression of the cell polarity protein Par6 and an activated small GTPase (Cdc42 or Rac1) promotes focus formation in an aPKC-dependent manner (Qiu et al., 2000). Thus aPKC expression can induce some but not all aspects of the transformed phenotype. There is some evidence that the two aPKC isoforms may play distinct roles in oncogenesis *in vivo*. PKC ζ has recently been shown to inhibit Ras-induced lung tumorigenesis *in vivo*, and PKC ζ was also previously reported to inhibit the survival signaling protein, Akt, in breast cancer cells (Galvez et al., 2009; Mao et al., 2000). In contrast, overexpression of PKC ι is sufficient to induce transformation of human non-small cell lung cancer cell lines and their tumorigenicity *in vivo*, and is required for colon carcinogenesis induced by Ras or overexpression of PKC β II (Murray et al., 2004; Murray et al., 2009; Regala et al., 2005a; Regala et al., 2005b). In addition, PKC ι is overexpressed and the *PKC ι* gene is amplified in a majority of primary human NSCLC tumors and serous ovarian cancers (Eder et al., 2005; Regala et al., 2005b). The evidence that PKC ι is a human oncogene and a potential target for anti-cancer therapeutics has recently been reviewed (Fields et al., 2007).

The PKC ι isoform is tyrosine phosphorylated by the non-receptor tyrosine kinase c-Src in PC12 cells (Wooten et al., 2001). NGF treatment also induced endogenous PKC ι kinase activity in a Src-dependent manner in these cells. Upon NGF treatment, Src and PKC ι co-immunoprecipitated in a signaling complex with the neurotrophin receptor, TrkA. In addition, purified c-Src phosphorylated and activated PKC ι *in vitro*. Thus PKC ι is a substrate for Src in NGF-responsive PC12 cells, and this tyrosine phosphorylation activates PKC ι kinase activity. It has also been reported that aPKC ι/λ undergoes Src-mediated tyrosine phosphorylation on pre-Golgi vesicular clusters and that this tyrosine phosphorylation promotes its association with Rab2 and glyceraldehyde-3-phosphate dehydrogenase (Tisdale and Artalejo, 2006).

Src activity is significantly elevated in many human epithelial cancers, and Src inhibitors are potential anti-cancer therapeutics (Tsao et al., 2007; Tuhackova, 2005). Elevated Src activity appears to be particularly important in tumor cell migration and invasion (Frame, 2004; Hiscox et al., 2006; Leupold et al., 2007). The observation that aPKC is tyrosine phosphorylated and activated by cellular Src suggested that some aspects of Src-dependent tumorigenesis may be dependent on aPKC activity. Transformation by retroviral Src (v-Src) is a well-characterized model for cell transformation. To determine the significance of aPKC activity in transformation by v-Src, we examined the phenotype of v-Src-transformed fibroblasts following inhibition of aPKC. We report here that aPKC activity is elevated in some v-Src transformed cells, and that aPKC activity is required for migration and invasion of v-Src transformed fibroblasts. Tyrosine phosphorylation of aPKC λ is required for its ability to promote cell invasion. The dependency of matrix invasion on aPKC function results, at least in part, from a requirement for aPKC activity for podosome assembly, protease secretion and matrix degradation.

Materials and Methods

Cell Culture and Cell lines

Cells were grown in Dulbecco's Modified Medium (DMEM) plus 10% calf serum. For growth in low serum, cells were washed in PBS and transferred to DMEM plus 0.5% calf serum. NIH3T3 cells stably expressing v-Src were generated by infection of NIH3T3 cells with pBabe-

Hygro (vector) or pBabe-Hygro-v-Src retroviruses harvested from the ψ 2 viral packaging cell line; hygromycin-resistant clones were isolated and screened for v-Src expression by immunoblot. Three clones were selected for further study: clone 2.11, designated here clone 1; clone 2.10, designated here clone 2; and clone 3.39, designated here clone 3. NIH3T3 cells expressing ER:v-src (SrcER cells) (McMahon, 2001) were washed in PBS and grown for 48 h in Opti-MEM reduced serum medium without phenol red (Gibco); 1.0 μ M 4-hydroxytamoxifen was added for a further 24 h to induce v-Src activity. To generate v-SrcER 3T3 cells stably expressing kinase-inactive PKC ζ (K281W), the cells were transfected with pCMV5 vector expressing Flag-tagged rat PKC ζ (K281W) (gift of Alex Toker, Harvard University) using Lipofectamine 2000 (Invitrogen); G418-resistant clones were isolated and screened for stable kinase-inactive PKC ζ expression by immunoblotting. NIH-3T3 cells transformed by Src 527F were obtained from Sara Courtneidge. Transient transfections were carried out with Lipofectamine 2000 transfection reagent (Invitrogen) according to the supplier's protocol. Small hairpin RNAs (shRNAs) were introduced into v-Src NIH-3T3 clone 1 cells using an Amaxa Nucleofector and the manufacturer's protocol for 3T3 cells.

Constructs

v-Src (Tian and Martin, 1997) was subcloned into pBabe-Puro. A full length human PKC ζ cDNA obtained from S. Ohno was tagged with an N-terminal Hemagglutinin tag by PCR subcloning into a pCDNA3.1-derived mammalian expression vector (pCAN-HA) obtained from Onyx Pharmaceuticals. The PCR subcloning was accomplished by generating a full-length PKC ζ PCR product containing an EcoRI restriction site in the N terminus and a XhoI restriction site in the C terminus using PKC ζ full-length 5'Forward Primer (aaaccggaattcgaaggagcggcgccgcgtc) and PKC ζ full-length 3'Reverse primer (aaaccgctcgagtcacaccgactctcggtggacag). pCMV5 His-tagged mouse PKC ζ and PKC λ were obtained from S. Ohno. pCMV5 Flag-tagged rat PKC ζ KD (K281W) was provided by Alex Toker and contains an inactive ATP-binding site (Romanelli et al., 1999). Small-hairpin DNA oligonucleotides generated to target mouse PKC ζ mRNA (target sequence: ggtgcagacagagaac) and mouse PKC λ (target sequence: ggtgcagacagagaagc) or a scrambled sequence (gatttcgagtcgtcttaa) were sub-cloned into pSuper GFP Retro (Oligoengine) according to the supplier's instructions or into a plasmid RFP/H1 encoding mCherry fluorescent protein (gift of M. Frohman, SUNY, Stony Brook). pCMV5 human PKC ϵ and PKC ϵ Y325F were obtained from M. Wooten, Auburn University, Auburn.

Antibodies and Reagents

Commercial reagents were purchased as follows: PKC ζ C-20 and H-1 rabbit polyclonal antibodies (which recognize both aPKC isoforms) from Santa Cruz Biotechnology; PKC ζ / λ anti-Phosphothreonine 410 rabbit polyclonal antibody and anti-PKC λ -specific antibody (Cat. No 610176) from Transduction Laboratories; anti-cortactin antibody and anti-phosphotyrosine 4G10 mouse monoclonal antibody from Upstate Biotechnology; anti-PKC ϵ -specific antibody for podosome co-localization from Santa Cruz Biotechnology (H-76); anti- β -tubulin antibody and 4-Hydroxy-Tamoxifen (98% Z-isomer) from Sigma; HRP-conjugated anti-mouse and anti-rabbit secondary antibodies from Cell Signaling Technologies; SU6656 from Calbiochem®; PKC ζ myristoylated pseudo-substrate peptide (Myr-SIYRRGARRWRKL) and myristoylated PKC η pseudo-substrate peptide (Myr-TRKRERAMRRRVHEING) from Biosource; rhodamine-conjugated phalloidin, Alexa Fluor® 488 and 546 goat anti-mouse and goat anti-rabbit secondary antibody conjugates and FITC-gelatin from Molecular Probes; anti- α -tubulin antibody and anti-Flag®M2 Antibody from Sigma; and anti-pericentrin antibody from Covance.

Transformation Assays

Soft agar assays were performed by suspending 5,000 cells of each 3T3 clone in soft agar (0.35%) and incubation for 12 days at 37°C. Colonies were visualized by staining with 0.5 ml of 0.005% Crystal Violet for 1 h and the number of colonies was counted using a dissecting microscope. Focus assays were performed by seeding 5×10^5 cells of each 3T3 clone onto 6 cm plates and incubation in medium containing 10% fetal calf serum for 2 weeks. The medium was changed every 4 days, and on day 14, the cells stained with 0.5% crystal violet in 95% ethanol for 1 min to visualize foci. Growth in low serum was monitored by seeding 7.5×10^4 cells of each 3T3 clone onto 6 cm plates and incubation in medium containing 0.5% fetal calf serum for 4 days. The cells were counted every 24 h.

Lysate preparation and Immunoblotting

Cells were lysed in mammalian lysis buffer (100 mM NaCl, 40 mM Hepes pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1.0% Triton X-100) containing protease inhibitors as described (Webb et al., 2000). Cell lysates were harvested on ice by scraping, and insoluble cellular material was pelleted at 10,000 rpm in an Eppendorf microfuge for 10 min at 4 °C. Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to an Immobilon-P transfer membrane (Millipore). Membranes were incubated with primary antibodies and then visualized with the appropriate secondary antibodies conjugated with horseradish peroxidase and SuperSignal® West Pico Chemiluminescent Substrate System (Pierce).

Immunoprecipitation

Cells were lysed and harvested in mammalian lysis buffer containing protease inhibitors as above and supplemented with a phosphatase inhibitor cocktail (final concentrations, 10 mM NaPO₄, 10 mM NaF, 0.1 mM β-glycerophosphate, 1 μg/ml phosphatidylserine and 1mM Na₂(VO₄)₃). 800 μg to 1.0 mg of cleared cell lysates were pre-cleared with normal immunoglobulin (2 μg) and protein A (for mouse monoclonal antibodies) or G PLUS (for rabbit antibodies) conjugated sepharose beads (20 μl) resuspended (50% by vol) in PBS (Santa Cruz Biotechnology) rotating for 1 h at 4°C. Beads were removed by brief centrifugation, and antibody (10 μl PKCζ C-20, 5 μl 4G10) and protein A or G PLUS sepharose beads (40-60 μl) were added. Immunoprecipitations were carried out on a rotating platform at 4°C overnight. Beads were washed 3 times in 1 ml of mammalian lysis buffer, and after the last wash, beads were re-suspended in 20-40 μl Laemmli Sample Buffer.

Kinase assays

To assess the phosphorylation of aPKC by Src *in vitro*, kinase reactions with purified proteins were performed with 0.1, 0.5 or 1.0 μg of recombinant PKCζ (Biosource) alone or with 0.1 μg of recombinant active Src (Upstate). Recombinant proteins were re-suspended in 50 μl Phosphorylation Buffer (20 mM tris-HCl pH7.5, 5 mM MgCl₂, 1 mM EGTA) containing 25 μM ATP. Reactions were performed at 30°C for 30 min and terminated by the addition of sample buffer. Samples were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-phosphotyrosine antibody. To assess the activity of aPKC *in vivo*, the enzyme was immunoprecipitated from 800 μg – 1 mg of cell lysate with 10 μl anti-PKCζ (C-20) antibody. Beads were washed 3 times with Mammalian Lysis Buffer, and once with Phosphorylation Buffer. One half of the beads was used to immunoblot with anti-aPKC (C-20) antibody to determine IP efficiency. The other half was re-suspended in 50 μl buffer containing 75 μM PKCε peptide substrate (Biosource), a preferred substrate for aPKC (Kazanietz et al., 1993) and 25 μM [γ-P³²]ATP (specific activity 2 Ci / mmole). Kinase reactions were performed for 30 min at 30°C with occasional agitation and terminated by cooling on ice and addition of 10 μl of 0.5 M EDTA pH 7.2. The beads were removed by centrifugation and 20 μl of

supernatant was spotted directly onto squares of P81 phosphocellulose paper. The paper squares were washed 2 times for 1 h with 1.0 L of 1% H_3PO_4 and once with 100 μl acetone, then allowed to dry completely. ^{32}P was quantified by scintillation counting.

Migration and Invasion Assays

Wound-healing assays were performed by plating $1.5 \times 10^6 - 2.0 \times 10^6$ cells onto 6-well plates containing sterilized coverslips on the day prior to wounding. Cells were scratched with a sterile pipette tip, and cells were allowed to migrate for the times indicated. The polarity of the cells bordering the wound was determined by centrosome and microtubule staining. Cells were fixed in 100% methanol and stained using antibodies against α -tubulin and pericentrin. The percent of bordering cells polarized into the direction of the wound was calculated by counting the number of cells along the wound border that contained the centrosome in the wound forward-facing quadrant of the cell. Chemotaxis and invasion assays were performed using BioCoat™ Matrigel Invasion Chambers (BD Biosciences) that contain a membrane with 8.0 μm pores with (invasion chamber) or without (chemotaxis chamber) Matrigel Basement Membrane Matrix. 2.5×10^5 cells suspended in medium supplemented with 0.5 % calf serum was added to the top of the chambers, and medium containing 10% calf serum was added to the bottom chamber as chemoattractant. Cells were allowed to migrate or invade for 23 hours. Chambers were washed with PBS and the cells on the surface of the membrane opposite to that to be counted were removed with cotton-tipped swabs. Cells on the bottom (or top where indicated) surface of the membrane were counted following fixation with 4% para-formaldehyde and staining with Vectashield® mounting medium containing 1.5 $\mu\text{g/ml}$ 4'6-diamidino-2-phenylindole (DAPI). The number of cells that migrated or invaded was determined by counting the number of cells per field at 20 $\times$ or 40 $\times$ magnification using fluorescence microscopy. To determine the percent of attached cells that migrated or invaded, both the top and bottom surfaces of the filter were counted, and the average number of cells per field on the bottom surface was divided by the average total number of cells on the top plus bottom surfaces. In experiments where cells had been transiently transfected with kinase-inactive PKC ζ or a vector encoding a shRNA against PKC ζ and λ , transfected cells on the top and bottom surfaces of the membrane were identified either by staining with H-1 anti-PKC ζ antibody or by visualization of the vector-encoded GFP; the ratio of transfected cells on the bottom surface to transfected cells on the top surface was then determined.

Immunofluorescence

Cells growing on glass coverslips were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature. After 3 washes in PBS + 1% Tween-20 (PBST), cells were permeabilized in PBST for 10 min to 1 hr, then incubated in PBST containing 1% BSA for 30 minutes at room temperature to block non-specific binding. Primary antibody was added to cells at 1:100 dilution overnight, rotating at 4°C, except for anti-cortactin antibody which was added at 1:400 dilution and incubated for 3 h at room temperature. After 3 washes with PBST, the appropriate secondary antibodies were added at 1:500 for 30 min, rotating at room temperature. Coverslips were washed 3 times in PBST and mounted on slides with Vectashield® mounting medium containing DAPI.

In situ zymography

Coverslips coated with FITC-gelatin for in situ zymography were prepared as in (Berdeaux et al., 2004). 5×10^4 cells were plated onto FITC-gelatin coated coverslips in 6-well plates containing 10% serum and allowed to degrade the gelatin for 2 h. Cells were fixed in 4% para-formaldehyde and stained with rhodamine-phalloidin to visualize the actin cytoskeleton, and with H-1 anti-PKC ζ or anti-GFP antibodies to visualize the transfected cells.

Results and Discussion

Activation of aPKC by v-Src

To determine the effect of v-Src expression on aPKC kinase activity, aPKCs were immunoprecipitated from three clones of 3T3 cells stably expressing similar levels of v-Src (Fig. 1a), using an antibody that recognizes both aPKC isoforms, PKC ζ and PKC λ , and immune complex kinase assays were performed using a peptide substrate. There was significant clonal variation in aPKC kinase activity (Fig. 1a). Clone 1 exhibited the highest level of aPKC activity, clone 2 exhibited a moderately elevated level of activity, and clone 3 exhibited no increase in activity over two independently-derived vector control clones. In clones 1 and 2 the Src-specific inhibitor, SU6656 caused a dose-dependent decrease in aPKC activity (Fig. 1b). We conclude that v-Src expression can result in an elevation in aPKC activity, but that other cellular factors can affect the ability of v-Src to regulate aPKC activity.

PKC ι is tyrosine phosphorylated and activated by c-Src (Wooten et al., 2001). To determine whether PKC ζ is also a substrate for activated Src *in vitro*, kinase assays were performed using purified, baculovirus-expressed PKC ζ and activated c-Src (Y527F). Incubation with activated c-Src increased the level of PKC ζ tyrosine phosphorylation (Supplementary Fig. 1a). In COS cells endogenous aPKCs exhibited tyrosine phosphorylation in cells transiently expressing v-Src but not in cells expressing kinase-inactive v-Src (Supplementary Fig. 1b). In 3T3 clone 2 stably expressing v-Src, which showed moderately increased aPKC activity, aPKCs displayed elevated tyrosine phosphorylation that was sensitive to inhibition by the Src inhibitor, SU6656 (Supplementary Fig. 1c). We also observed a rapid but transient increase in aPKC tyrosine phosphorylation upon activation of a regulatable v-Src-estrogen receptor fusion protein (Supplementary Fig. 2d). Thus v-Src phosphorylates aPKCs both *in vitro* and *in vivo*.

aPKC activation by v-Src correlates with tyrosine phosphorylation but not with activation loop phosphorylation

There was significant clonal variation in the level of aPKC tyrosine phosphorylation (Fig. 2a). Interestingly, the level of aPKC activity appeared to correlate with the degree of aPKC tyrosine phosphorylation. Clone 1 displayed the highest levels of aPKC tyrosine phosphorylation and activation, and clone 2 intermediate levels, while clone 3 displayed little or no aPKC tyrosine phosphorylation and no significant increase in aPKC activity. All three clones express comparable levels of v-Src (Fig. 1a) and all three display an elevation in total phosphotyrosine level that is partially reversed by SU6656 (Supplementary Figs. 1 and 2c). Furthermore, only the two clones with elevated aPKC activity and tyrosine phosphorylation showed a decrease in aPKC kinase activity when incubated with SU6656 (Fig. 2b). We conclude that aPKC activation by v-Src correlates with its tyrosine phosphorylation. The explanation for these clonal differences is unknown: one might speculate that there are differences in localization that affect the ability of v-Src to access its substrate, or that there might be clonal differences in the activity of a phosphotyrosyl-protein phosphatase specific for aPKCs.

Tyrosine phosphorylation of PKC ι at tyrosine 256 promotes nuclear localization of PKC ι (White et al., 2002). However in all three clones of v-Src transformed fibroblasts examined, we found wild-type aPKC to be predominantly cytoplasmic, regardless of tyrosine phosphorylation (unpublished data). Mutation of a tyrosine residue (Y325) near the catalytic loop of PKC ι , inhibits the ability of Src to activate over-expressed PKC ι (Wooten et al., 2001). However the mechanism or mechanisms by which tyrosine phosphorylation of aPKCs regulates their activity remains largely unknown. It could result in a direct effect on enzymatic activity or an alteration in the ability of aPKCs to interact with binding partners such as Rab2 (Tisdale and Artalejo, 2006). These possibilities remain to be explored. The role of tyrosine phosphorylation of aPKC in matrix invasion is discussed further below.

Activation loop phosphorylation of aPKC on threonine 410 by PDK1 is required for aPKC kinase activity. However transient transfection of v-Src in COS cells did not increase the level of aPKC phosphorylation by PDK1 (Fig. 2c). Furthermore there was no increase in Thr410 phosphorylation even in v-Src transformed clone 1 (Fig. 2d). Thus, aPKC activation by v-Src correlates with tyrosine phosphorylation but not with activation loop phosphorylation.

aPKC activity contributes to transformation by v-Src

All three v-Src clones examined were morphologically transformed, irrespective of the level of aPKC activity, indicating that elevated aPKC activity is not required for morphological transformation (Fig. 3a). To further examine this question, v-Src transformed clone 1 was incubated with a myristoylated aPKC pseudo-substrate inhibitor previously been shown to inhibit aPKC function (Muscella et al., 2003; Sun et al., 2005). The cells became flatter with an increase in actin stress fibers following incubation with the aPKC pseudo-substrate (Fig. 3b). This change was only clearly apparent in the clone 1 cells (data not shown), presumably because these cells also display the highest aPKC activity.

We also examined the ability of all three clones to grow in soft agar, proliferate in low serum and form foci (Fig. 3c-e). We found no significant differences in the ability of the different v-Src-transformed cells to grow in soft agar, a classical criterion of transformation. Although there were differences between the clones in their ability to grow in low serum and form foci, these differences did not correlate with aPKC activity, and this variation presumably reflects the well-known genetic heterogeneity of NIH-3T3 cells. All three clones were able to degrade extracellular matrix in *in situ* zymography assays, but clone 3 exhibited a somewhat reduced capacity to degrade the matrix (Fig 3f), suggesting that aPKC may be involved in the invasiveness of v-Src transformed cells (see below).

aPKCs are required for migration and invasion of v-Src transformed cells

aPKCs have previously been reported to be important in regulation of cytoskeletal architecture and cell migration (Etienne-Manneville and Hall, 2001; Muscella et al., 2003; Soloff et al., 2004; Sun et al., 2005). aPKCs have also been reported to be required for cell invasion of human non-small cell lung cancer cells (Frederick et al., 2008). To investigate the role of aPKC function in migration and invasion of v-Src transformed fibroblasts, we examined the effect of the myristoylated aPKC pseudo-substrate inhibitor on migration of Src-transformed clones 1 and 3 across uncoated membranes in Boyden transwell chambers and on their ability to invade through Matrigel-coated membranes (Fig. 4, panels a,b). As a control, the cells were incubated with a PKC η myristoylated pseudo-substrate inhibitor. Incubation with the aPKC pseudo-substrate inhibitor resulted in a dose-dependent decrease in the migration and invasion of Src-transformed cells (Fig. 4a). Non-transformed cells migrated more rapidly than the v-Src transformed cells (Fig. 4b); it is possible that the v-Src transformed cells are less migratory under these conditions because they are significantly less adherent to the substrate. The migration of the non-transformed cells was not inhibited by either the aPKC or the PKC η pseudo-substrates. In contrast, the migration of both the v-Src transformed clones 1 and 3 was inhibited when the cells were incubated with the aPKC pseudo-substrate inhibitor but not when incubated with the PKC η pseudo-substrate inhibitor (Fig. 4b). The number of cells attached to the upper surface of the membrane was not affected by incubation with the aPKC pseudo-substrate inhibitor (Supplementary Fig. 3). The aPKC pseudo-substrate also inhibited the ability of both clones 1 and 3 to invade extra-cellular matrix (Fig. 4b). There was a less pronounced reduction in cell invasion when these clones were incubated with the PKC η pseudo-substrate inhibitor. Non-transformed cells were not invasive under any conditions, at least within the time-frame of this experiment. We conclude, first, that Src-transformed cells are dependent on aPKC function for both migration and invasion, and second, that this

dependence is exhibited both by cells in which aPKC is elevated and cells in which it is not elevated.

To confirm these conclusions, we used pools of 3T3 cells stably co-expressing a regulatable form of v-Src, Src fused to the estrogen receptor (SrcER), and a kinase-inactive form of PKC ζ previously shown to function as a dominant negative (Diaz-Meco et al., 1993) (Fig. 4c); activation of SrcER by 4-hydroxytamoxifen leads to morphological transformation (Supplementary Fig. 4) The percentage of cells expressing kinase-inactive PKC ζ on the bottom surface of both the migration and invasion membranes was much lower than the percentage on the top surfaces, indicating that expression of dominant-negative PKC ζ inhibits both migration and invasion.

In v-Src-transformed cells both aPKC isoforms, PKC ζ and PKC λ , promote migration and invasion

There are two isoforms of aPKC in mammalian cells, PKC ζ and PKC λ/ι . However immunodepletion with an antibody that specifically recognizes PKC λ removed most of the total aPKC pool from lysates of transformed clone 1 cells (Fig. 5b), suggesting that PKC λ is the most abundant isoform in these cells; a slightly smaller fraction was immunodepleted from clone 3 cells (Fig. 5b). To determine whether one or both aPKC isoforms are important for migration and invasion of v-Src-transformed cells, clone 1 cells were transfected with plasmid DNA encoding small-hairpin RNAs (shRNAs) directed against the two isoforms or with plasmid DNA encoding kinase-inactive PKC ζ ; in COS cells the shRNAs directed against PKC ζ and PKC λ caused a significant reduction in the expression of co-transfected His-tagged protein (Fig. 5 a). When shRNAs for both PKC ζ and PKC λ were expressed in Src-transformed clone 1, there was a significant reduction in both cell migration and the ability of the cells to degrade extracellular matrix. The reduction in cell migration and invasion observed when both shRNAs were expressed was less than that observed when the cells expressed kinase-inactive PKC ζ (Fig. 4c), possibly because the shRNA clones do not completely eliminate the expression of the aPKCs isoforms. Knockdown of PKC ζ expression alone did not significantly inhibit cell migration or invasion, while knockdown of PKC λ alone resulted in a modest reduction in cell migration and a significant reduction in invasion. We conclude that both isoforms contribute to migration and invasion, with PKC λ playing a more important role. This may be because there are intrinsic differences between PKC ζ and PKC λ , or because the PKC λ isoform is expressed at higher levels in these cells (Fig. 5b), or both.

PKC ι was reported to be tyrosine phosphorylated by Src *in vitro* at several residues, and one residue, tyrosine 325, was shown to be critical for Src activation of PKC ι *in vivo* (Wooten et al., 2001). In order to determine whether PKC λ tyrosine phosphorylation is important for its role in cell invasion, wildtype human PKC ι or PKC ι Y325F was expressed in clone 1 cells along with the small hairpin targeted against mouse PKC λ (Fig. 5e). Wild-type PKC ι was able to rescue invasion of clone 1 cells expressing the hairpin targeted against PKC λ , but PKC ι Y325F was not, suggesting that tyrosine phosphorylation of PKC ι is critical for its role in cell invasion.

aPKCs are required for polarization of migrating v-Src transformed fibroblasts

aPKCs are required for the establishment of cell polarity during migration of mammalian cells (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000). We determined the effect of aPKC inhibition on cell polarization in the v-Src-transformed cells by examining the effects of the aPKC and PKC η pseudo-substrates on the distribution of the microtubule organizing center (MTOC) protein pericentrin in cells migrating into a wound in the monolayer. The aPKC inhibitor resulted a significant reduction in the percentage of cells polarized in the direction of migration in all three v-Src transformed clones examined, while there was no effect with the

PKC η pseudo-substrate (Fig. 6a). Non-transformed cells expressing empty vector also displayed a reduced ability to polarize when incubated with the aPKC pseudo-substrate, although the reduction was less than that observed in the v-Src transformed cells. 3T3 cells co-expressing the kinase-inactive PKC ζ with a regulatable form of v-Src, SrcER, also demonstrated a reduced ability to polarize into the wound (Fig. 6b). These observations suggest that v-Src transformed fibroblasts show an increased dependence on aPKC function for the establishment of cell polarity during directed migration. Since this dependence is also exhibited by clone 3, in which aPKC activity is not elevated, we conclude that this increased dependence is not reflective of increased kinase activity. It is possible that non-transformed cells, which are more adherent, may obtain additional polarity cues from the substrate or ECM, leading to a lower requirement for aPKC activity during migration than that exhibited by transformed cells. It seems likely that this increased dependence on aPKC function for polarization accounts, at least in part, for the requirement for aPKC function in migration of v-Src-transformed cells.

PKC λ promotes extracellular matrix degradation in v-Src transformed fibroblasts

In the mesenchymal mode of invasion exhibited by Src-transformed fibroblasts, the ability to invade the extracellular matrix is dependent on proteolytic degradation of the matrix. We observed that incubation of Src-transformed clone 1 cells with the aPKC pseudo-substrate led to a large decrease in the number of cells digesting FITC-gelatin in in situ zymography assays (Fig. 7a). Clone 1 cells expressing kinase-inactive PKC ζ , also exhibited a significant decrease in the fraction of cells capable of degrading gelatin (Fig. 7b). When PKC λ expression was suppressed by a shRNA, the percentage of cells degrading gelatin was also significantly decreased (Fig. 7c). In contrast, there was no decrease in the percentage of cells degrading gelatin when PKC ζ expression was suppressed, and co-expression of shRNAs targeted against PKC λ and PKC ζ did not reduce gelatin degradation more than knockdown of PKC λ alone. This suggests that PKC λ is the primary aPKC involved in extracellular matrix degradation in clone 1 v-Src transformed fibroblasts, consistent with the prior finding that PKC λ is the more abundant isoform in these cells. aPKCs have previously been shown in other systems to regulate the expression of matrix metallo proteases (MMP -1,-3,-9 or -10) which digest extracellular matrix (Frederick et al., 2008; Hussain et al., 2002; Urtreger et al., 2005). However, we could not detect any changes in expression of these MMPs in clone 1 cells upon inhibition of aPKC (data not shown).

v-Src transformed fibroblasts adhere to the substrate through invasive adhesions known as podosomes or invadopodia, actin-rich structures on the ventral surface that secrete proteases which digest the extracellular matrix. Upon aPKC inhibition there was a decrease in the number of podosomes when the cells were cultured in fetal calf serum (Fig. 8a). To determine which aPKC isoform is responsible for podosome assembly, clone 1 cells were transiently transfected with small hairpins directed against PKC ζ , PKC λ or both isoforms and stained for cortactin to visualize podosomes (Fig. 8b). Expression of the hairpin DNA directed against PKC λ significantly inhibited podosome assembly in these cells, while expression of the hairpin directed against PKC ζ did not cause a reduction in podosome assembly. This suggests that the PKC λ isoform is the more important isoform for podosome assembly. We also observed the co-localization of endogenous aPKCs to podosomes in Src transformed cells that display enlarged podosomes (Fig. 8c). Both the anti-aPKC antibody that recognizes both aPKC isoforms and the anti-PKC λ -specific antibody detected aPKC at the podosomes. This suggests that aPKCs may regulate podosome assembly by directly modifying proteins at the site of podosomes in v-Src transformed cells. One possible aPKC substrate is Smurf, an E3 ubiquitin ligase specific for activated Rho, which is known to bind aPKC at the leading edge of migrating cells (Wang et al., 2003). Another aPKC substrate is MEK5, which activates the MAP kinase ERK5 (Diaz-Meco and Moscat, 2001). We have recently shown that Src activation of ERK5

promotes podosome formation by limiting the extent of Rho activation (Schramp et al., 2008). Therefore, it is possible that aPKC may also limit the level of activated Rho in podosomes. The mechanism by which aPKCs regulate podosome assembly and the secretion of ECM proteases merits further investigation.

Conclusions

There is substantial evidence that aPKCs are important regulators of tumor formation and carcinogenesis *in vivo*. Recently, PKC ϵ has been described as an oncogene in human non small cell lung cancer (NSCLC) cells, in which expression of a kinase-inactive PKC ϵ inhibited tumor growth in nude mice, while expression of a constitutively active PKC ϵ promoted tumor growth (Regala et al., 2005a). PKC ϵ is frequently overexpressed in human breast cancers and this overexpression correlates with invasive ductal carcinoma (Kojima et al., 2008). In addition, PKC ϵ is overexpressed and the *PKC ϵ* gene is amplified in a majority of primary human NSCLC tumors and serous ovarian cancers (Eder et al., 2005; Regala et al., 2005b). PKC ϵ is also critical for human glioblastoma cell chemoresistance (Baldwin et al., 2006). On the other hand, there is conflicting evidence for the role of PKC ζ in cancer *in vivo*. Increased expression of PKC ζ has been detected in the majority of human metastatic prostate cancers, and PKC ζ was shown to be commonly mutated in colon cancers, by two independent studies (Rhodes et al., 2007; Wood et al., 2007). However, it has recently been shown that PKC ζ can function as a tumor suppressor in mice, whereas deletion of *PKC ζ* leads to increased lung carcinogenesis (Galvez et al., 2009). We show here that tyrosine-phosphorylated PKC λ is required for invasion by certain v-Src-transformed cells, whereas PKC ζ is not required. This difference may reflect differences in the relative levels of expression of the two isoforms, but it may also reflect intrinsic differences in the ability of the two isoforms to promote invasion. Tyrosine phosphorylation may increase aPKC enzymatic activity (Wooten et al., 2001) or alter its ability to interact with binding partners such as Rab2 (Tisdale and Artalejo, 2006). It should be noted however that aPKCs are neither activated nor tyrosine phosphorylated in clone 3 transformed cells, but these cells are nevertheless invasive and basal aPKC activity is required for cell invasion. Thus aPKCs appear to play multiple roles in the invasive properties of Src-transformed cells.

In summary, the findings reported here indicate that the migratory and invasive phenotypes of Src-transformed cells and their ability to degrade the extracellular matrix through podosome assembly are aPKC-dependent. Since Src activity is elevated in many epithelial cancers, and since Src activity also correlates with increased invasive and metastatic ability, our findings provide new insights into how aPKCs promote the invasiveness of transformed cells and further evidence that aPKCs may represent potential targets for anti-cancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Clonal variation of aPKC kinase activity in v-Src transformed cells **(a)** aPKCs were immunoprecipitated from lysates of 3T3 cell clones expressing v-Src (clones 1 - 3) or empty vector and the immunoprecipitates were incubated with an aPKC peptide substrate and $\text{Mg}^{2+}[\gamma\text{-P}^{32}]\text{ATP}$. Values represent the fold increase in kinase activity (vector = 1). Below, anti-Src immunoblots of the same lysates. **(b)** Immune complex kinase assays were performed as in (a) on lysates of v-Src-3T3 cells (clone 2) that had been incubated with different concentrations of the Src inhibitor SU6656 for 24 hours.

Fig. 2. aPKC activity correlates with v-Src-dependent tyrosine phosphorylation but not phosphorylation of Threonine 410. **(a)** 3T3 clones expressing v-Src were serum-starved for 48 h and incubated with 1 μM or 10 μM of SU6656 for 24 h. aPKC was immunoprecipitated from cell lysates, and immunoblotted with anti-phospho-tyrosine (top) or anti-aPKC (bottom) antibody. **(b)** aPKC immune complex kinase assays were performed on lysates of 3T3 clones treated as in **(a)**. Values shown represent the percent inhibition in aPKC kinase activity by 10 μM SU6656. **(c)** aPKC was immunoprecipitated from COS cells transiently expressing v-Src or kinase-inactive v-Src (v-SrcKD) and immunoblotted with anti-pThr410 antibody (top) or anti-aPKC antibody (bottom). **(d)** aPKC was immunoprecipitated from 3T3 cells stably

expressing v-Src (clone 1) or empty vector and immunoblotted with anti-pThr410 antibody (top) or anti-aPKC antibody (bottom).

Fig. 3.

aPKC activity contributes to morphological transformation by v-Src. **(a)** 3T3 clones were fixed and stained with rhodamine-phalloidin to visualize actin stress fibers. **(b)** v-Src transformed clone 1 cells were grown in medium containing 10 % fetal calf serum for 24 h, incubated with 40 μ M aPKC pseudo-substrate for 7 h, and then stained as (a). **(c)** 5,000 cells of each 3T3 clone were grown in soft agar (0.35%) for 12 days, and the numbers of colonies were counted. **(d)** 5×10^5 cells of each 3T3 clone were seeded onto 6 cm plates and grown in medium containing 10% fetal calf serum for 2 weeks. Foci were stained and counted. **(e)** 7.5×10^4 cells of each 3T3 clone were seeded onto 6 cm plates and grown in medium containing 0.5% fetal calf serum for 4 days. The cells were counted every 24 h. **(f)** Cells of each 3T3 clone were seeded onto

coverslips coated with FITC-gelatin and allowed to degrade the gelatin for 2 h. The cells were then fixed, stained with rhodamine-phalloidin and DAPI, and the percentage of cells that degraded the gelatin was determined by fluorescence microscopy. In this and subsequent figures, asterisks indicate significant differences by t test: * < 0.05, ** < 0.01, *** < 0.001).

Fig. 4.

Migration and invasion by v-Src transformed cells requires aPKC activity. **(a)** 3T3 cells expressing v-Src (clone 1) were seeded onto trans-well chambers with Matrigel (invasion) or without Matrigel (migration) and the extent of migration and invasion determined as described under Materials and Methods. **(b)** 3T3 cells expressing v-Src (clones 1 and 3) or empty vector (ϕ) were seeded onto migration (top) and invasion chambers (bottom) with or without 5 μ M pseudo-substrate inhibitor for aPKC ζ or PKC η . Cells were counted on either the top of the filters (to determine number of attached cells) or on the bottom surface of the filters (to determine the number of cells migrating or invading). Values shown are the percent attached cells migrating or invading. **(c)** 3T3 cells expressing SrcER and transfected with kinase-

inactive PKC ζ were pooled after 3 weeks of drug selection and seeded onto migration and invasion chambers containing 4-OH-Tamoxifen. After 23 h cells on the bottom and top surfaces of the filters were fixed and stained with anti-aPKC antibody to detect the cells expressing kinase-inactive PKC ζ or with DAPI to detect both expressing and non-expressing cells. The percentage of cells expressing kinase-inactive PKC ζ was determined for both top and bottom surfaces of the filters and the ratio of the two percentages was compared to the ratio of total cells on the top and bottom surfaces for cells expressing empty vector (100%).

Fig. 5.

PKC ζ and PKC λ isoforms are required for migration and invasion of v-Src transformed cells. (a) COS cells were transfected with plasmids encoding His-tagged PKC ζ (left) or PKC λ (right) and shRNAs directed against PKC ζ (left) or PKC λ (right) or empty vector (ϕ). Cell lysates were immunoblotted with anti-His antibody. (b) PKC λ was immunoprecipitated from lysates of v-Src transformed 3T3 cells (clones 1 and 3) using varying amounts of anti-PKC λ antibody. The immunoprecipitates and immunodepleted supernatants were then immunoblotted using an anti-aPKC antibody that recognizes both PKC ζ and PKC λ . (c) and (d) v-Src transformed 3T3 cells (clone 1) were transfected with plasmid DNAs encoding GFP-tagged shRNAs directed

against PKC ζ , PKC λ or a scrambled shRNA. The transfected cells were seeded onto migration **(c)** and invasion **(d)** chambers, and allowed to migrate and invade as described in Fig. 4c. Cells on the bottom and top surface of filters were fixed stained with DAPI, and the percentages of GFP-expressing cells on the top and bottom surfaces determined by fluorescence microscopy. Values shown represent the percentage of GFP-expressing cells on the bottom relative to the top surface of the membrane (scrambled shRNA = 100). **(e)** Clone 1 cells were transiently transfected with mCherry-tagged small hairpins targeted against PKC ζ , PKC λ , both isoforms, or empty vector with or without co-transfection with human PKC ι wildtype or PKC ι Y325F. 24 h post-transfection, cells were seeded onto migration and invasion chambers for 23 h and cells were stained for PKC ι and visualized as described in **(c)** and **(d)**.

Fig. 6.

Inhibition of aPKC reduces cell polarization during monolayer wound healing in v-Src transformed cells. **(a)** Monolayers of v-Src transformed 3T3 cells or cells expressing empty vector were scratched then incubated with either PKC ζ (ζ) or PKC η (η) pseudo-substrate and allowed to fill the wound for 10 h. Cells were then fixed and stained with anti-pericentrin antibody (MTOC marker), anti-tubulin antibody and DAPI. The graph represents the percent of cells on the immediate border of the wound that displayed the MTOC in the wound-forward facing quadrant of the cell. **(b)** Wound-healing assays were performed with 3T3 cells stably expressing SrcER and kinase-inactive PKC ζ or SrcER only. Cells were seeded in medium containing 10% calf serum and 4-OH-Tamoxifen. Cells were allowed to grow for 7.5 h after wounding and then fixed and stained with anti-pericentrin antibody. The percent of cells on the immediate border of the wound that displayed pericentrin on the wound forward-facing quadrant of cell was then determined.

Fig. 7.

Inhibition of PKC λ reduces extracellular matrix degradation. (a) v-Src transformed 3T3 cells (clone 1) were incubated with or without aPKC pseudo-substrate for 24 h, then seeded onto coverslips coated with FITC-gelatin and allowed to degrade the gelatin for 2 h. The cells were then fixed, stained with rhodamine-phalloidin and DAPI, and the percent of cells that degraded the gelatin was determined by fluorescence microscopy. (b) v-Src transformed 3T3 cells (clone 1) were transfected with plasmid DNA encoding kinase-inactive PKC ζ (PKC ζ KD) or empty vector. After 48 hours, the transfected cells were seeded on FITC-gelatin coated coverslips as in (a), and the percentage of the transfected cells degrading gelatin determined. (c) v-Src transformed 3T3 cells (clone 1) were transfected with plasmid DNAs encoding mCherry fluorescent protein and shRNAs directed against PKC ζ (ζ) and/or PKC λ (λ) or plasmid DNA encoding a scrambled shRNA sequence. The transfected cells were then plated on FITC-gelatin coated coverslips as in (b), and the transfected cells were visualized by staining with anti-aPKC antibody.

Fig. 8.

aPKC is localized at podosomes and is required for podosome assembly in v-Src transformed cells. (a) v-Src transformed 3T3 cells (clone 1) were grown in medium supplemented with 10% fetal calf serum with or without 40 μ M aPKC pseudo-substrate for 7 h. The cells were fixed and stained with rhodamine-phalloidin and anti-cortactin antibody to visualize podosomes. (b) v-Src transformed clone 1 cells were transfected with small hairpins targeted against PKC ζ , PKC λ or both isoforms. The cells were stained with anti-cortactin antibody and podosomes were visualized by microscopy. (c) transformed NIH-3T3 cells expressing activated Src 527F were stained with anti-cortactin antibody, phalloidin and either aPKC antibody or PKC λ specific antibody. Immunofluorescence was visualized by confocal microscopy to determine co-localization of aPKC and podosomes.